STUDIES ON THE BIOLOGY AND CHEMISTRY OF THE GULF OF MAINE

III. Bacteriological Investigations of the Sea Water and Marine Bottoms

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In former expeditions undertaken for the study of marine life, the occurrence, abundance, and activities of the bacteria were either left out entirely, or were based upon the examination of samples of water brought back many days and frequently months after they were collected. The bacteriological activities in the sea bottom were frequently completely overlooked. In view of the fact that the total numbers of the bacteria as well as the relative abundance of specific forms will change rapidly soon after the samples of water and mud are exposed to surface temperature, due either to a change in environmental conditions or to a change in food supply, it is quite essential that the collected samples should be subjected to immediate investigation. As a result of these considerations, the laboratory on the "Atlantis," during the cruise made in August, 1932, was so outfitted that it was possible to carry out the bacteriological studies within a few minutes or at most hours, after the samples of water, bottom mud, or sand were brought up from their respective depths.

Because of the extensive hydrographic and biological investigations which have been carried on in the Gulf of Maine, this region is particularly well adapted to a study of the relative abundance and activities of marine bacteria. Such a study was further aided by the fact that other biological and chemical investigations were carried on simultaneously on this cruise. The locations of the various stations at which samples were taken during this cruise are described in the first paper of this series.[2] Of the eight stations studied, five were in the Gulf of Maine proper and the remaining three in the shallow waters over Georges

[1] Contribution No. 10 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station.

[2] Rakestraw, Norris W., 1933. Chemistry of the Waters of the Gulf of Maine in August, 1932. *Biol. Bull.*, **64**: 149.

Bank. A detailed description of the methods used in these investigations will be presented later, when the bacteriological results of this expedition will also be compared with those obtained from waters and sea bottoms near shore. Only a brief summary of the investigations carried out on and in connection with this cruise of the "Atlantis" will be reported here. An attempt will be made to interpret the results of these investigations in the light of the chemical conditions of the waters and nature of the marine bottom, as well as to correlate the results of the bacteriological investigations with the results of the chemical and planktonological studies made by the other investigators on this cruise.

Abundance of Bacteria in Sea Water and in the Sea Bottom

The relative numbers of bacteria in the sea are usually determined by means of the plate method. This method has numerous limitations and can at best give only a very approximate idea of the actual abundance of the bacteria, both in the water and in the sea bottom. Although new methods have been introduced recently, consisting in direct staining of the bacteria in the water, after they have been concentrated by filtration or centrifuging, these methods are still in an experimental stage and were not used on this expedition. If the various limitations of the plate method are kept in mind and if broad generalizations are avoided, the results obtained even by this procedure can be made to throw considerable light upon the relative abundance and nature of the bacterial population of the sea.

The numbers of bacteria were determined in the water from the different stations at three different depths, namely, the surface, 50 meters, and the bottom. At one station, water from two additional depths of 30 and 100 meters was also investigated. The surface samples were taken with ordinary sterilized and evacuated glass tubes provided with a sealed glass tip which was broken about one foot below the surface of the water. Deeper samples were taken in heavier-walled glass tubes having a specially constricted neck to prevent the rubber stopper being forced into the tube by the pressure of the water. The inlet tube in this case was made of a capillary glass tube having a 1 mm. bore. Tubes of this type were used successfully to a depth of 330 meters but the lower limit of their usefulness has not yet been determined. Mud samples were taken by means of a sampler consisting essentially of a brass tube carrying a weight sufficient to force it some distance into the mud when the bottom is reached. Into the brass tube is fitted a glass tube having a check valve attached to its upper end. This allows water to stream through the tube during its descent. While raising the sampler the

valve closes and prevents any downward pressure upon the mud core within. By sterilizing the glass tube before use it is possible to eliminate any bacterial contamination of the mud sample other than the contamination of the walls of the glass tube by the water passing through it while descending. This may be easily eliminated by taking the sample to be studied from the center of the core and discarding the surface which has been in contact with the glass tube.

The water was plated undiluted and diluted 10 times, while the mud was plated in dilutions of 1:10, 1:100 and 1:1000. Previous experiments, which will be reported elsewhere, have shown that the following medium is satisfactory for the development of marine bacteria, capable of growing on the ordinary plate:

Agar	15 grams
Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water	1000 cc.

TABLE I

Abundance of bacteria in sea water. Total numbers in 1 cc. of water.

Station Number		1329	1330	1331	1332	1333	1334	1335	1336
Total depth of water, *meters*		256	207	230	346	230	71	74	64
Depth of sample, *meters*	Surface	10	3	4	2	380	16	92	6
	30			0					
	50	1	1	2	1	2			
	100			1					
	Bottom	6	0	1	1	3	25	41	27

The plates were incubated at air temperature and the colonies counted at the end of 7 and 10 days. All the samples of water and mud were plated out within a few minutes after being taken.

The total numbers of bacteria in the water, as determined by the plate method, are given in Table I. With respect to their bacterial content, the stations may be divided into two groups, the first containing the five deep water stations and the second the three shallow stations. With the exception of the surface water of Station 1333, the bacteria in the water of the first group, namely, Stations 1329 to 1333 inclusive, were uniformly so extremely low in number, that it is difficult to draw reliable conclusions in regard to their distribution. It would seem, however, that the bacteria were slightly more numerous at the surface than in the lower depths of water. There was no appreciable variation between

the different other depths sampled, either at a single station or between different stations. Neither was there any appreciable variation in the bacterial content of the surface water of the different stations of this group. The very high content of the surface water of Station 1333 was probably due to the sample inadvertently being taken near floating seaweed or the introduction of some other contamination. The water samples from the lower levels of this station resembled those from the other deep water stations in their bacterial content.

The bacteria in the water of the second group of stations were uniformly much more numerous than in the water from the deeper stations. In view of the other results to be reported below, this may be ascribed to the higher phytoplankton content of this water. In two of the three stations the bottom water samples were considerably higher in bacteria than the surface samples. One must keep in mind the fact that the bottom of the last group of stations was sandy, while the bottom of the first four stations was mud. To what extent the deposits at the bottom of the ocean directly affect the bacterial content of the water for any very great distance above, except in turbulent waters, still remains to be determined. Because of the stray of the sampling cable from the perpendicular and the rise and fall of the ship with the waves, it is not possible to secure water samples with the apparatus used nearer than 2 or 3 meters from the bottom. The results, therefore, cannot be taken as indicating the bacterial content of the layer of water in contact with the mud bottom. It is possible to secure water samples nearer the bottom at shallow stations, as in the case of the second group. This, together with the fact that the water at these stations is rather turbulent, may indicate the transfer of sufficient nutrient material from the bottom deposit to the water immediately above to support the greater number of bacteria found in these waters.

The more uniform distribution of the bacteria in the water at the shallower stations is also correlated with the even distribution of the diatoms. This is probably due to the stronger vertical mixing of the waters, as shown by the even distribution of temperature and salinity.

The results reported above agree with those of various other investigators in indicating that the numbers of bacteria in sea water are relatively low and quite uniform in distribution. In an effort to secure further information concerning the presence of bacteria in sea water, the association of these organisms with the plankton forms was investigated. By towing with plankton nets samples of both zoöplankton and phytoplankton were collected. A number 20 silk net was used, which left out the nannoplankton. The zoöplankton consisted largely of copepods, while the phytoplankton was made up almost entirely of

diatoms with a few *Ceratium* cells present. This material was collected in the glass jar at the bottom of the net and portions of it immediately plated out. The numbers of bacteria in the various tow waters are given in Table II.

The water containing the diatom plankton is extremely rich in bacteria. It was not possible to make determinations of the actual quantity of plankton present in the samples. Apparently the zoöplankton has fewer bacteria associated with it than the phytoplankton. Dr. Gran estimated that the sample obtained from Station 1336 contained 11,900 planktonic organisms per liter of tow water, of which 11,100 were diatoms. It is of considerable interest to compare the relative numbers of diatoms in the tow and in the sea water at this station with the numbers of bacteria in the tow water and in the sea water. In the case of the zoöplankton (Station 1331), there were 225 times as many bacteria in 1 cc. of the tow as in 1 cc. of the corresponding water, thus giving a ratio of the bacteria in the tow to the bacteria in the water of 225 : 1.

TABLE II

Abundance of bacteria in tow water. Numbers in 1 cc. of tow.

Station No.	Type of plankton	Total bacteria	Agar-decomposing bacteria	Percentage agar-decomposing bacteria
1331	Zoöplankton	890	0	0
1335	Diatom plankton	36,700	2,100	5.7
1336	Diatom plankton	37,500	2,500	6.7

In the case of the diatom plankton, the ratio between the bacteria in the tow and the bacteria in the water (taking the average of the surface and bottom samples) was 500 : 1 and 2,270 : 1, for Stations 1335 and 1336 respectively. It is important to note that Dr. Gran found that the ratio for the respective numbers of diatoms in the tow and in the water was about 1,667 : 1. This indicates that a definite parallelism exists between the bacteria and the diatoms in the water and in the plankton. The actual existence of such relations is substantiated by the fact that the bacteria from the diatom tow showed a considerable abundance of agar-liquefying organisms. While one encounters only infrequently such organisms in the free water, usually only very near shore, and in the mud, they were found to make up 5.7 and 6.7 per cent of the total bacterial flora in the plankton tow, as determined by the agar plate method. It is interesting to note that the bacteria found in the copepod-plankton did not include any agar-liquefying forms.

It is quite possible, therefore, that bacteria exist only to a very

limited extent in the free water of the sea, but are largely attached to the plankton organisms, living upon the dead members of the plankton or upon the excretion products of the cells, upon the cell membranes, especially the mucilaginous substances secreted by certain algæ, etc. One need not, of course, imagine that if such an associative growth exists, the numbers of higher plankton forms and bacteria will always be parallel. One can readily imagine that the bacterial maximum may be attained after the diatom and copepod maximum. This has been brought out in an experiment carried out in cooperation with Dr. Gran and described by him elsewhere, in which the numbers of diatoms and of bacteria were determined simultaneously in artificial cultures of diatoms in sea water (Table III). The rise in numbers of both groups of organisms appears to have been directly related. The increase in bacterial numbers was much greater proportionately than that of the diatoms. When the multiplication of the latter reached a maximum,

TABLE III

Comparative numbers of diatoms and bacteria in diatom cultures. Numbers in 1 cc. of culture water.

	Original water	After 3 days growth	After 7 days growth	After 12 days growth
Diatoms	177	1,026	178	165
Bacteria	346	20,600	22,800	13,900

the numbers of bacteria continued to rise further, while the numbers of diatoms began to fall. This may be noted at the 7-day period when the diatoms had dropped to their original level, while the bacterial numbers were still increasing. This is to be expected since the death of the diatoms provides a source of food for the saprophytic bacteria. The numbers of bacteria dropped slowly and, within the course of the experiment, did not fall to the level of the original water as did the diatoms, indicating a greater resistance of bacteria to the artificial culture conditions.

Because of the large numbers of bacteria capable of decomposing agar found associated with the diatoms of Stations 1335 and 1336, it was of interest to follow their behavior in this experiment. In the original water the number of agar-decomposing bacteria was of the order of 2 or 3 cells per cubic centimeter. At the end of the 3-day culture period this figure had risen to 50 cells per cubic centimeter. At none of the later periods did any agar-decomposing bacteria appear on

TABLE IV

Abundance of bacteria in the sea bottom. Numbers in 1 gram of mud or sand, dry basis.

Station Number	1329	1330	1331	1332	1335	1336
Surface	5,400	4,700	830	3,000	1,280	670
10 cm	1,720	1,700	140	290		

the plates. The disappearance of these bacteria may be ascribed to their overgrowth by other forms better adapted to the cultural conditions. This is shown by the fact that while the colonies on the plates from the original water indicated the presence of a considerable variety of bacterial forms, in the last plating from the cultures only one or two types remained.

The terrigenous bottom deposits of the Gulf of Maine contain varying amounts of organic matter originating partly from the organisms

TABLE V

Presence of Anærobic Bacteria in the Marine Bottom

Medium		Solution				Agar			
Dilution		10		100		10		100	
Station No.	Depth of mud	1	2	1	2	1	2	1	2
1329	0–30 cm.	+	+	+	0	5*	2*	0*	2*
	30–60 cm.	+	0	+	0	12	2	4	8
	60–90 cm.	+	0	0	0	+	+	0	0
1330	0–30 cm.	+	+	+	0	30	18	10	8
1331	0–30 cm.	0	0	0	0	?	?	0	0

+ Indicates growth and gas formation.
* Figures = numbers of colonies in the agar shake-tube.

living in the sea bottom and sinking from the water above, but probably more largely from the organic matter present in a partly decomposed state in the sediments brought in from land. These forms of organic matter furnish the energy for the growth of the bacteria in the marine bottom. The total organic matter content in the mud and sand bottoms of the stations investigated is reported later, while a chemical study of the organic matter in sea bottoms or marine humus is reserved for another publication.

Mud samples for bacteriological investigation were obtained from Stations 1329 to 1332. The bottom at Station 1333 was made up of

pebbles and no sample could be secured. The sand bottom of Stations 1334 to 1336 could not be sampled by the procedure outlined above because of the sand slipping from the tube as it was withdrawn from the bottom. However, at two of these stations sand samples were obtained by means of a Petersen grab. The numbers of bacteria found in these samples, both at the surface of the sample and at a depth of 10 cm., in the case of the mud bottoms, are given in Table IV.

TABLE VI

*Numbers of bacteria in sea water and in mud, as shown by the dilution method, using gelatin as the medium for bacterial development.**

Station	Depth†	Dilution	Water	Mud
		cc.		
1329	Surface	0.1		++
1329	Surface	0.01		++
1329	Surface	0.001		++
1329	10	0.1		++
1329	10	0.001		+
1330	Surface	0.01		++
1330	Surface	0.001		+
1330	Surface	0.0001		0
1330	10	0.01		++
1330	10	0.001		0
1331	Surface	0.01		++
1331	Surface	0.001		++
1331	Surface	0.1	+	
1331	Surface	0.01	0	
1331	30	0.1	0	
1331	30	0.01	0	
1331	50	0.1	+	
1331	50	0.01	0	
1331	100	0.1	+	
1331	100	0.01	+	
1331	215	0.1	0	

* + indicates growth after 3 weeks incubation; ++ indicates very good growth.
† Depth in water, is, in meters, from surface of water; depth in mud is, in centimeters, from surface of mud.

The bacterial numbers in the surface layer of the mud were found to vary from 830 to 5400 per gram of dry mud. These figures for the mud may be compared with the values of 670 and 1280 per gram of dry sand at Stations 1335 and 1336. The two samples of mud (1329 and 1330) which contain more organic matter contain also more bacteria; variations which occur in the different mud samples may be ascribed to differences in organic matter content or to physical variations.

The total number of organisms decreases rapidly with the depth of

mud. Thus at Station 1329, at a depth of 10 cm., the number of bacteria is only 1,720 as compared to 5,400 at the surface. The decrease is even more noticeable in the mud of Station 1332. The bacteria do not disappear rapidly, however, below the surface layer of the mud, but extend to considerable depths, as shown by the data for the presence of anærobic organisms (Table V). These bacteria were determined by the dilution method, using liquid medium kept under anærobic conditions, and by the shake-tube method, using agar medium. The results indicate the widespread occurrence of anærobic bacteria near the surface of the mud and their presence to depths as great as 90 cm. below the surface.

There is no doubt that the numbers of bacteria found in sea water and in the sea bottom by the plate method represent only a fraction of the total numbers of bacteria. This is due to the limitations of the method, whereby only a part of the bacterial population develops on the plate into colonies. In order to check this, several samples of water and mud were diluted and 1 cc. portions of the various dilutions added to sterile portions of 10 per cent gelatin in sea water. The results presented in Table VI show that whereas the plate method gave only 1 to 4 cells in 1 cc. of water from Station 1331, the dilution method with gelatin as a medium gave positive growth in some instances with 0.1 cc. of water and in some even with 0.01 cc. of water, indicating that at least 10 to 100 bacterial cells were present in 1 cc. of the water. In the case of the mud samples, abundant positive growth was obtained in most instances with 0.001 gram.

Results of bacterial investigation of the water and bottom samples of the Barents sea, recently reported by Butkewitch,[3] also bring out the fact that the dilution method may give 10 to 100 times as many bacteria as the plate method. The direct microscopic method gives even larger numbers, usually a thousand times greater than those obtained by the plate method. This difference is due to the fact that not only living but also dead bacteria are counted by the microscope, while many bacteria unable to develop on artificial media are seen with the microscope.

Occurrence of Specific Groups of Bacteria in the Sea

A number of bacteria active in certain specific processes, which are no doubt of considerable importance in the metabolism of the sea, were demonstrated both in the sea water and in the sea bottom. It is sufficient to mention here the presence of ærobic and anærobic nitrogen-fixing bacteria, of nitrifying bacteria, of agar-liquefying and cellulose-decom-

[3] Butkewitch, W. C. Method of Bacteriological Investigation and Certain Data on the Distribution of Bacteria in the Water and Bottom of Barents Sea. Trans. Russian Oceanogr. Inst., 2: No. 2, 1932.

posing bacteria, of nitrate-reducing bacteria, of various spore-forming and non-spore-forming bacteria, and of a number of heterotrophic ærobic as well as anærobic bacteria. Some of these organisms will be discussed in detail later, while others need only be mentioned here in passing.

At least three types of cellulose-decomposing bacteria were demonstrated to be present in the sea. They belong largely to the groups described by Winogradsky as *Cytophaga, Cellvibrio,* and *Cellfalcicula.* They produce yellow and orange pigments or no pigment at all. They were found in the water from Station 1329 and to a less extent in the mud of the same station, but they were especially abundant in the tow water collected at Station 1336. This phenomenon may suggest the probability that these organisms, as well as the agar-liquefying bacteria, also present abundantly in the diatom tow, as pointed out previously, are not found to any great extent in the free water or in the mud, but develop in close connection with the growth of the plankton organisms, especially the phytoplankton; one would expect that the latter would offer a logical substrate for the growth of such bacteria. Some of these bacteria were able to attack both cellulose and agar, while others attacked cellulose alone and still others grew upon agar alone.

The agar-liquefying bacteria are represented in the sea by a number of different groups. They produce yellow, pink, or brownish pigments or no pigment at all. Most of them are ærobic, while some are anærobic. A detailed description of these organisms will be published in a later study. Chitin-decomposing bacteria have also been demonstrated in the sea water but these organisms have as yet been insufficiently studied.

It is commonly assumed that the sea harbors very few spore-forming bacteria. This may be true of the water itself; the sea bottom, however, especially the mud bottom, was found to harbor large numbers of such bacteria. The spore-forming bacteria could best be demonstrated in the nitrogen-free media inoculated with various dilutions of the mud samples. Many of the spores were found to have the typical appearance of *Clostridium,* so that one came to expect the development of these organisms in the nitrogen-free media inoculated with mud. This organism was accompanied by other bacteria, some of which were spore-forming. Many spore-forming bacteria were found abundantly in the calcium acetate-sodium nitrate medium used for the study of nitrate-reducing bacteria.

Anærobic bacteria were demonstrated by the use of liquid and solid media sealed with vaseline. Gas formation in the liquid media and colony development in the shake-tubes were taken as evidence of the development of these organisms. The ordinary peptone-glucose medium

was used for this purpose. The cultures were inoculated with material from the mud profiles (Table V). The formation of colonies in the anærobic cultures brought out the fact that at least several hundred anærobic cells are present in each gram of moist mud, even to a depth of 60 cm. of mud.

Occurrence of Nitrogen-fixing Bacteria in the Gulf of Maine

The problem of nitrogen-fixation in the sea has always aroused considerable interest due to the importance of this phenomenon in marine metabolism. If it is true, as some oceanographers assume, that the sea produces as much plant and animal life as a similar area of land, the amount of nitrogen required for the synthesis of these numerous forms of life is quite enormous, since the nitrogen content of the various representatives of marine life varies from 0.7 per cent in the case of certain algæ, such as *Fucus*, to 10 per cent in the case of various animals which range from the lowly copepods to the large fish and mammals.

By the use of silica-gel plates and liquid media containing a layer of sand, the presence in the sea of the two important groups of non-symbiotic nitrogen-fixing bacteria, namely the aerobic *Azotobacter* and the anærobic *Clostridium*, was definitely demonstrated. The occurrence of organisms in the sea water and sea bottom of the Gulf of Maine, capable of growing on nitrogen-free liquid media, is brought out in Table VII. The nitrogen-fixing organisms were found both in the surface waters and in the marine bottom. Samples of water taken from Station 1331 at depths of 30, 100, and 215 meters gave largely negative results.

A detailed description of the methods used in this study as well as the nature of the organisms found and their nitrogen-fixing capacity will be reported in another publication. It suffices here to say that a nitrogen-free medium containing a carbon source (glucose, mannitol, salts of organic acids), a source of phosphate and organic iron was used. In the case of the plates, this medium was added to silica-gel prepared in large Petri dishes (15 cm. in diameter); it was then subjected to dialysis in tap water, and the plates finally soaked for a few minutes in boiled sea water. Each plate contained 2 grams of carbon source. The liquid medium was placed in large test tubes with a layer of sand on the bottom and sterilized.

The silica gel plate inoculated with mud from Station 1329 gave a

TABLE VII

Distribution of bacteria capable of growing on nitrogen-free media in ocean waters and marine bottoms

Station No.	Depth†	Dilution in cc. or grams	Sea Water						Marine Bottom					
			Mannitol			Glucose			Mannitol			Glucose		
			Growth*	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡	Growth	Gas	Organisms‡
1329	Surface	1.0	T, P	0	Bac.	P	0	Rods	−	−	—	−	−	−
1329	Surface	0.1	T	0	Bac.	T, P	0	−	T, P	+	Bac.	T	+	Bac.
1329	Surface	0.01	0	0	0	0	0	0	P	+	Bac.	T	−	−
1330	Surface	1.0	−	−	−	−	−	−	T, P	+	l.r.	−	+	sm.r.
1330	Surface	0.1	−	−	−	−	−	−	T	+	−	P, T	+	−
1331	Surface	1.0	T, P	−	sm.r.	T	0	sm.r.	−	−	−	−	−	−
1331	Surface	0.1	−	−	−				T	+	Bac.	T	+	Bac.
1331	Surface	0.01	−	−	−				T	+	Bac.	T	+	Bac.
1331	30	1.0	−	−	−	−	−	−						
1331	50	1.0	T, P	0	0	T	0	−						
1331	100	1.0	P	0	0	T	0	−						
1331	215	1.0	0	0	0	T	0	−						
1332	Surface	0.1	−	−	−	−	−	−	P	+	Bac.	T	+	l.r.
1332	10	0.1	−	−	−	−	−	−	−	−	−	T	+	−
1333	Surface	0.1	T, P	0	−	T, P	0	−	−	−	−			
1333	Deep	0.1	−	−	−	T, P	0	sm.r.						
1334	Surface	0.1	T	0	−	T	0	−	−	−	−			
1334	Deep water	0.1	T, P	0	sm.r.	−	−	−	−	−	−			
1335	Surface	1.0							T, P	+	sm.r.	T, P	+	l.r.
1336	Surface	1.0							P	−	sm.r.	P	+	sm.r.

* P = pellicle formation, T = turbidity.

† Depth in water, in meters, is from surface of water; depth in mud, in centimeters, is taken from surface of mud.

‡ Bac. = spore-forming rods; sm.r. = small rods; l.r. = large rods.

typical development of *Azotobacter chroococcum*. This organism was readily isolated and grown in pure culture. On nitrogen-free sea water media, with glucose or mannitol as a source of energy, it fixed four to six milligrams of nitrogen for one gram of carbohydrate in 21 days.

Occurrence in the Gulf of Maine of Bacteria Capable of Oxidizing Ammonium Salts to Nitrites

If there is any one group of bacteria that has attracted the greatest attention of oceanographers and of bacteriologists alike, it is the group of nitrifying bacteria, or those organisms which produce nitrites from ammonium salts and nitrates from nitrites. A review of the various theories proposed to explain the occurrence of nitrite and nitrate in the ocean would in itself require considerable space. It suffices here to bring out only the results of the "Atlantis" expedition, to enable one to draw some very definite conclusions. It is important to emphasize in this connection that negative results do not necessarily mean the absence of the specific organisms but merely that the possibility exists that the medium used for demonstrating these organisms and the conditions essential for their development were not particularly favorable. Positive results are, however, much more important.

Of the two processes leading to the formation of nitrate, the first one, namely the oxidation of ammonium salts to nitrite, is usually considered to be more specific and more important, hence this process was more emphasized during this cruise.

Preliminary investigations of the water taken near shore brought out the fact that nitrite formation can take place in a sea water medium if proper conditions are provided. Three methods were used for this purpose: (1) Sand and nutrient salts—$CaCO_3$, $(NH_4)_2SO_4$, K_2HPO_4—were added, in proper concentration to flasks and these were sterilized; the fresh sea water taken from the various stations and at the different depths was introduced directly into these flasks. (2) In another set of flasks sand taken from Georges Bank was used in a fresh state and water taken from some of the stations was added. (3) In a third experiment, sea water, sand, and chemicals were placed in flasks, sterilized, and inoculated with mud from the various stations. The results obtained on the "Atlantis" cruise are presented in Table VIII. These results prove definitely that nitrite-forming bacteria are completely absent in the free sea water or are present there only in very limited numbers, while the sea bottom contains an abundance of such organisms. In the case of the mud, the presence of nitrifying bacteria was demonstrated at quite appreciable depths. This tends to demonstrate that nitrite formation and probably also nitrate formation take

place in the sea bottom. The nitrite and nitrate ions will then diffuse into the water and will be gradually brought upwards by ocean currents, etc. Near the surface of the water, where active photosynthesis takes place, the nitrates tend to disappear due to the fact that they are rapidly consumed by the phytoplankton organisms.

Occurrence in the Sea of Bacteria Capable of Reducing Nitrates to Nitrites and to Atmospheric Nitrogen

The presence in the sea of bacteria capable of reducing nitrates has attracted considerable attention from oceanographers, largely as a result of the hypothesis proposed by Brandt,[4] who attempted to base the whole

Table VIII

Presence of nitrifying bacteria in sea water and sea bottom

Nitrite-forming bacteria in water			Nitrite bacteria in sea bottom		
Station No.	Depth of water	Nitrite formation in 20 days	Station No.	Depth of mud	Nitrite in 11 days
	meters			*cm.*	
1329	Surface	−	1329	0–30	+++
1329	Bottom	−	1329	30–60	++
1330	Bottom	−	1329	60–90	trace
1331	Surface	−	1330	0–30	++
1331	30	trace	1330	30–60	trace
1331	50	+	1330	60–90	trace
1331	100	−	1331	0–35	trace
1331	215	+	1336	Sand	+
1331	Bottom	−			
1332	Surface	+			
1332	50	+			
1332	100	+			
1333	Surface	trace			
1334	Surface	−			
1334	Bottom	trace			

system of marine metabolism on the occurrence and activities of these organisms. Preliminary studies in this laboratory have shown that when a liquid medium containing Ca-acetate, $NaNO_3$, and a layer of sand is inoculated with shore water or with mud, active nitrite formation will take place, accompanied by abundant gas evolution. When this medium was inoculated with mud, even in a dilution of 1:1000, the nitrate disappeared completely within 5 days. In other words, both the water and the mud close to the shore contain an abundance of organisms

[4] Brandt, K. Ueber den Stoffwechsel im Meere. Wiss. Meeresunters. Kiel, N. F., **4**: 213–230, 1899; **6**: 23–79, 1902; **18**: 185–429, 1916–1920; **19**: 251–253, 1919.

capable of reducing nitrates to nitrites, as well as of reducing the latter completely to atmospheric nitrogen.

For the investigations carried out on the "Atlantis" cruise, a medium similar to the above and containing 10 grams Ca-acetate, 1 gram $NaNO_3$, 0.5 gram K_2HPO_4, and a trace of iron per liter of sea water

TABLE IX

Relative abundance of nitrate-reducing bacteria in sea water

Station number	Depth of water	Dilution of water	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
	meters	*cc.*				
1329	Surface	1.0	Turbid	Large round cells and curved rods	−	+++++
1329	Surface	0.1	−	−	−	−
1329	Surface	0.01	−	−	−	−
1331	Surface	1.0	Turbid	Minute coccobacilli	−	+++++
1331	Surface	0.1	Slight turbidity	Minute coccobacilli	−	+++++
1331	Surface	0.01	−	−	−	−
1331	30	1.0	Turbid	Cocci and bacilli	−	+++++
1331	30	0.1	Turbid	Cocci and bacilli	−	+++++
1331	30	0.01	−	−	−	−
1331	50	1.0	Turbid	Cocci and bacilli	−	+++++
1331	50	0.1	−	−	−	−
1331	100	1.0	−	−	−	−
1331	100	0.1	−	−	−	−
1331	215	1.0	Slight turbidity	Long rods and cocci	−	+++++
1331	215	0.1	−	−	−	−
1333	Surface	0.1	Turbid	Small coccobacilli	−	+++++
1333	Surface	0.01	Turbid	Small coccobacilli	−	+++++
1333	Surface	0.001	Turbid	Small coccobacilli	−	+++++
1333	Bottom water	0.1	−	−	−	−
1333	Bottom water	0.01	−	−	−	−
1334	Surface water	0.1	−	−	−	−
1334	Surface water	0.01	−	−	−	−
1334	Deep water	0.1	−	−	−	+++++
1334	Deep water	0.01	−	−	−	−

was placed, in 25 cc. portions, in large test tubes containing a layer of sand. The medium was sterilized at 15 lbs. for 15 minutes. The tubes were inoculated immediately after the samples were taken, using various dilutions of water and mud. After two weeks incubation at air temperature, the cultures were examined microscopically and tested for nitrite (Tables IX, X).

Of all the cultures inoculated with water or with mud, only one gave positive gas evolution, namely, the one inoculated with 0.1 gram of mud from Station 1329. Positive growth of bacteria was always accompanied by a reduction of the nitrate to nitrite. This experiment tends to prove that the waters and the mud in the Gulf of Maine are able to reduce nitrates only to nitrites, but not to atmospheric nitrogen. In the case of the waters, nitrate reduction was active to a depth of 50 meters. Morphologically, the organisms developing in these cultures were found to differ considerably with the different inocula. Some were plain rods, others resembled *Azotobacter* cells, while still others were undeniably spores.

TABLE X

Relative abundance of nitrate-reducing bacteria in the mud

Station number	Dilution of mud	Growth of bacteria	Microscopic examination	Gas evolution	Nitrite formation
1329	0.1	Turbid	Spores	++	++
1329	0.01	Turbid	Few cells	−	++++++
1329	0.001	0	0	0	0
1330	0.1	Turbid	Oval rods, spores (?)	−	+++
1330	0.01	Turbid	Oval rods, spores (?)	−	++++
1330	0.001	Turbid	Oval rods, spores (?)	−	−
1330	0.0001	−	−	−	−
1331	0.1	Turbid	Spores	−	++++++
1331	0.01	Some turbidity	Spores	−	+++
1331	0.001	Some turbidity	−	−	+++
1331	0.0001	−	−	−	−
1332	0.1	Turbid	Spores, rods	−	++++++
1332	0.01	Turbid	Spores, rods	−	++++++
1332	0.001	−	−	−	−

EXPLANATION OF NITRATE CYCLE IN THE SEA ON THE BASIS OF THE OCCURRENCE OF AMMONIA-OXIDIZING AND NITRATE-REDUCING BACTERIA

The results of the investigations carried out in connection with this expedition prove beyond any doubt that the marine bottom, both sand and mud, contains bacteria capable of oxidizing ammonia to nitrite, and later to nitrate; the process of nitrate formation in the sea must, therefore, be considered as bacteriological in nature. The results also show that the sea water and marine bottom contain bacteria capable of reducing nitrate to nitrite, in the presence of sufficient energy material, in the form of readily available carbon compounds. The fact that marine humus cannot be used as such a source of energy, while the amount of available carbohydrate in the mud is very limited, tends to emphasize that this process in the sea is only of questionable importance.

The problem finally remains to establish whether the limited occurrence of nitrite in sea water is due to the oxidation of the ammonia by nitrifying bacteria or to the reduction of nitrate by other bacteria. There is no doubt that the oxidation takes place chiefly at the bottom of the ocean; further that while the formation of nitrite from ammonium salts, under proper conditions of culture and with bottom material as an inoculum, can be readily demonstrated, nitrate formation takes place, under laboratory conditions at least, at a much later date. Reduction of the nitrate can be expected to take place both in the sea water and in the sea bottom; however, this process requires a supply of organic matter as an available source of energy. This can only be obtained through the photosynthetic activities of the planktonic organisms. It is no mere coincidence, therefore, that the maximum nitrite formation corresponds well with the maximum oxygen content in the water or the maximum photosynthetic activities.

The following hypothesis suggests itself at this particular point: Decomposition of the organic nitrogenous compounds takes place in the sea water but largely on the sea bottom, with the result that the ammonia is then liberated. This ammonia is rapidly oxidized by specific bacteria living in the bottom to nitrite and later to nitrate. This nitrate remains in the sea bottom and is not reduced, due to a lack of available energy for the nitrate-reducing bacteria and not to a lack of such bacteria. The small amounts of ammonia found in the sea water originate from the decomposition of the plant and animal residues in the plankton and in the water. The nitrate formed in the bottom gradually diffuses into the water, where it remains as such. On reaching the zone of photosynthetic activities, this nitrate is either consumed by the phytoplankton or is reduced by the nitrate-reducing bacteria to nitrite, which may also be gradually consumed by the plants. Very little nitrate reduction to gaseous nitrogen or complete denitrification is possible under normal sea conditions. Reduction of nitrate to nitrite does not mean necessarily any loss of nitrogen from the cycle of life in the sea.

Abundance of Marine Humus in the Bottom of the Gulf of Maine

A series of mud samples was taken with the small glass tube from the first four stations and from one sand station, in order to determine the abundance as well as the variability in distribution of the organic matter in the sea bottom. The methods of analysis, as well as the importance of these results in determining the origin and nature of the organic matter or the marine humus in the sea will be discussed in a

later publication. The organic matter is reported in terms of carbon and nitrogen. In order to calculate the organic matter content of the mud or sand from the carbon data, the factor 1.724, which has found extensive application in soil investigations, may be used, for reasons discussed elsewhere.

TABLE XI

Distribution of marine humus in the sea bottom, as shown by the carbon and nitrogen content. Percentage of dry material.

Station No.	Sample No.	Carbon content	Nitrogen content	C/N
1329	1	2.46	0.284	8.7
1329	2	2.50	0.290	8.6
1329	3	2.48	0.290	8.5
1329	4	2.46	0.282	8.7
1329	5	2.37	0.278	8.6
1329	Average	2.45	0.285	8.6
1330	1	2.65	0.304	8.7
1330	2	2.66	0.284	9.4
1330	3	2.62	0.306	8.6
1330	4	2.70	0.294	9.2
1330	5	2.81	0.288	9.7
1330	Average	2.69	0.295	9.1
1331	1	1.58	0.156	10.1
1331	2	1.54	0.148	10.4
1331	3	1.55	0.144	10.8
1331	4	1.36	0.133	10.2
1331	5	1.29	0.133	9.7
1331	Average	1.46	0.143	10.2
1332	1	0.82	0.072	11.4
1332	2	1.20	0.096	12.5
1332	Average	1.01	0.084	12.0
1335	1	0.084	0.008	10.5
1336	1	0.12	0.009	13.3

The results reported in Table XI show that while the variation in the organic matter content is very small in some of the stations, it is quite appreciable in others. These results point definitely to the conclusion that determinations based upon a single sample may frequently give quite inaccurate results.

The distribution of marine humus in the mud profiles taken from the various stations is given in Table XII. A heavy brass sounding tube was used for this purpose. The depth of the mud brought up by means of this corer ranged from 75 to 115 cm. As soon as brought up, the cores were divided into three or four sections and later each analyzed separately. It is important to remember, of course, that the mud was compressed by the sampler and that the actual depth of the mud was greater than that shown by the figures. The analyses of the total carbon and nitrogen were made on the materials which had been dried in an oven at 100° C. There has been some question, however, as to the influence of drying upon the changes in the nitrogen content of the mud.

TABLE XII

Relative distribution of marine humus with the depth of the mud profile. On basis of dry material.

Station No.	Depth of mud	Carbon content	Nitrogen content	C/N
	cm.	*per cent*	*per cent*	
1329	0–30	2.58	0.290	8.7
1329	30–60	2.45	0.250	9.8
1329	60–90	2.32	0.238	9.75
1330	0–30	2.74	0.296	9.25
1330	30–60	2.75	0.254	10.8
1330	60–90	2.60	0.236	11.0
1331	0–35	1.58	0.140	11.3
1331	35–65	1.64	0.130	12.6
1331	65–90	1.61	0.128	12.6
1331	90–115	1.57	0.126	12.4
1331	90–115 (reddish mud)	1.04	0.080	13.0
1332	0–25	0.67	0.050	13.4
1332	25–50	1.12	0.092	12.2
1332	50–75	1.43	0.096	14.9

The samples obtained from Station 1329 were, therefore, analyzed for total nitrogen both in a moist and in a dry condition and both sets of results calculated on a dry basis (Table XIII). These results show that while in some instances there is no appreciable difference in the nitrogen content of the mud as a result of its preliminary drying in the oven, in other cases the difference obtained may be appreciable. All the analyses reported here were made on the oven-dried (60–90° C.) mud, due primarily to the ease of handling and to the greater uniformity of the samples.

Stations 1329, 1330, and 1331 show a gradual even if only a very limited decrease of the humus content with an increase in depth of mud,

especially on the basis of the nitrogen figures. This is accompanied by a gradual decrease in the nitrogen content of the humus. This is due either to the greater decomposition of the nitrogen complexes with the age of the humus or to a difference in the nature of the materials deposited at different times. One is struck, however, by the great relative uniformity of the organic matter in the mud throughout the whole profile. One exception to this is found in one of the samples from the 90–115 cm. layer of Station 1331. This section of the profile was distinctly different in color from the rest of the profile and even from the duplicate part of another profile, being much redder in color. This proves again that even at one and the same station the mud may not be very uniform; this was brought out in Table XI for the data obtained from this station.

In the case of Station 1332, there is an increase in the humus content with an increase in the depth of the profile, as shown by both the carbon and nitrogen figures. This is due entirely to a difference in the

TABLE XIII

Influence of drying of marine mud upon its nitrogen content. On basis of dry mud.

Depth	Moist mud analyzed	Dry mud analyzed
cm.	*per cent N*	*per cent N*
0–30	0.306	0.290
30–60	0.282	0.250
60–90	0.239	0.238

nature of the material deposited at different periods, the surface material being much coarser than the lower layers. The amount of humus in the sand bottoms is very limited, usually equivalent to about 0.1 per cent carbon on the basis of the dry material. In view of the fact that no apparatus has been devised as yet to enable one to take a deep core of the sand bottoms, it is impossible to state how this humus content changes with the depth of the bottom. No information is available concerning any differences that may exist in the chemical nature of the humus in the mud and sand bottoms.

On comparing the data of the humus content in the mud (Table XI) with those of the bacterial numbers (Table IV), one finds a certain definite correlation. This is especially true when one compares Stations 1329 and 1330 with a high humus content, on the one hand, with Stations 1331 and 1332 with a low humus content, on the other. One must keep in mind, of course, the fact that the numbers of bacteria were determined

either in the very surface layer of the mud or in the 10 cm. layer, while the organic matter content was determined in the total core which ranged in depth from 15 to 25 cm. The bacteria are present most abundantly in the uppermost surface layer, where the organic matter is of recent origin and no doubt different chemically from the organic matter in the lower depths of the mud. The surface layer of the mud can be looked upon as the layer most active bacteriologically. The same is probably true of the sand, where the relative abundance of bacteria is considerably greater than the relative humus content. The abundance of humus can, therefore, be looked upon as only one factor controlling bacterial numbers and activities.

Summary

A bacteriological survey has been made of the waters and bottom sediments in the Gulf of Maine and Georges Bank. The samples of water, plankton tow, and sedimentary material were taken under sterile conditions and subjected immediately to bacteriological analysis as soon as brought on board the "Atlantis."

The agar-plate method was used for the enumeration of the numbers of bacteria. This was supplemented to a limited extent by the dilution method. Various specific media were used to determine the distribution and relative abundance of certain groups of bacteria which are believed to take part in important marine processes.

The results obtained demonstrated the fact that the bacterial population of the sea can be divided into three groups on the basis of their habitat: (1) those forms which live in the sea bottom, especially in the surface layers; (2) those bacteria which live in the free water, this being possible only when the water contains in solution organic and inorganic substances which can serve as nutrients for the bacteria; (3) those bacteria which live largely upon or in association with the plankton organisms.

Sea water is a rather poor medium for the growth of bacteria, while the marine bottom is comparatively richer in the total number of bacteria capable of developing on the plate and in solution media. Mud bottoms contain more living bacterial cells than sand bottoms. However, the waters above the sandy bottom were found to contain many more bacteria than the waters above the mud bottoms. This may be due to the greater abundance of plankton organisms, especially diatoms, in the shallower seas with the sandy bottom, to the greater mixing of the waters, or to the greater absorption of bacterial cells by the mud bottom material than by the sand bottom.

The numbers of bacteria obtained by the plate method represent only a part of the bacterial population of the sea. This was shown by the fact that the dilution method, using gelatin as a medium for bacterial development, gave higher numbers than the plate method.

A decided parallelism was observed between the abundance of diatoms in the sea and abundance of bacteria. A comparatively large number of these bacteria were agar-liquefying organisms. In the artificial culture of diatoms, the numbers of bacteria were found to increase with the development of the diatoms. However, when the latter reached a maximum and began to die out, bacterial numbers did not diminish very rapidly; these bacteria seemed to be largely limited to a few specific types, the importance of which in marine processes still remains to be determined. These results seem to point definitely to the fact that the development of phytoplankton in the sea is accompanied closely by bacterial development. The bacteria feed upon the excretion products of the diatoms, algæ, and animal forms and probably upon these plankton forms themselves as soon as they die, thus bringing about their rapid disintegration and liberation of the nutrient elements in an available form.

Anærobic bacteria were found abundantly in the marine mud; these bacteria were present in the mud even at considerable depths. Their presence points to continued decomposition of the plant and animal debris of the ocean on and in the ocean bottom, even with an insufficient supply of oxygen.

The bacterial population of the sea was found to consist of a number of types, some of which take part in well-known processes which are of great importance in the metabolism of the sea, such as nitrogen-fixing, nitrite- and nitrate-forming, nitrate-reducing, cellulose-decomposing, agar-decomposing, chitin-decomposing, and many others. The importance of many common bacteria, both ærobic and anærobic, mostly non-spore-forming but also spore-forming, in marine processes still remains to be determined. The presence of nitrogen-fixing bacteria, comprising both the ærobic *Azotobacter* and the anærobic *Clostridium*, has been definitely demonstrated. One strain of the first group was isolated in pure culture and was found to be *Azotobacter chroococcum*, which fixed considerable quantities of nitrogen when grown on artificial culture media, with various carbon sources.

Bacteria capable of oxidizing ammonium salts to nitrites were found in the sea bottom, but only seldom in the sea water. For demonstrating the presence of these organisms in the sea, a medium containing a layer of sand and $CaCO_3$ and covered with a shallow layer of liquid containing an ammonium salt and the necessary minerals was used.

Bacteria capable of reducing nitrate were found abundantly both in the water, especially at the surface layers and the zone of photosynthetic activities, and in the sea bottom. However, these bacteria were able to reduce nitrate only to nitrite and not to nitrogen gas; only in one instance, namely, in the mud from the first station taken on this expedition, was there present an organism capable of bringing about the last process.

The cycle of nitrate in the sea is explained as follows: The nitrogenous constituents of the plant and animal residues in the sea are decomposed with the liberation of ammonia, largely in the sea bottom. The ammonia is oxidized in the sea bottom to nitrite and later to nitrate. The latter gradually diffuses into the waters. It is not reduced in the sea bottom or lower layers of water, largely because of a lack of available energy material necessary for the activities of the nitrate-reducing bacteria. In the zone of maximum photosynthetic activity such energy is available, hence nitrate-reduction may take place, but only to nitrite, since bacteria capable of reducing it to nitrogen gas are lacking under those conditions. The nitrite thus formed may also be assimilated by the phytoplankton and does not represent any loss of nitrogen in the sea.

The marine humus (total organic matter) content of the mud bottom in the Gulf of Maine is found to be more or less uniform in composition. This humus is best calculated from the total organic carbon. It was found that determinations based upon one sample may not give absolute evidence, since different samples may give considerable variation. The humus content usually decreases with the depth of the mud. In some stations, however, an increase in humus content with depth was observed. There is also a widening of the carbon-nitrogen ratio of the humus with an increase in depth. This points to a greater decomposition of the humus, especially of the nitrogenous complexes, with an increase in depth. An increase in humus content with depth of mud found in some stations merely points to the fact that the rate of deposition of the humus may vary considerably with different periods.

A definite parallelism was observed between the numbers of bacteria and abundance of humus in the mud.

A detailed review of the literature bearing upon the results presented in this report, as well as a detailed description of the methods used and a correlation of these results with the results of bacteriological investigations of the sea near shore, is reserved for a future publication.